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The Organizer-Associated Chick Homeobox Gene, *Gnot1*, Is Expressed before Gastrulation and Regulated Synergistically by Activin and Retinoic Acid

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***Gnot1*, a Not (for notochord) family homeobox gene, is expressed in the chick pregastrulation blastoderm. *Gnot1* expression in the epiblast is upregulated as the posteriorly derived hypoblast moves forward anteriorly to form a layer beneath it, which is of particular interest considering the known inductive role of the hypoblast in axis formation in the chick. Both activin and retinoic acid are able to activate *Gnot1* expression in cultured blastodermal cells and show a strong synergistic effect when applied in combination. Strong superinduction of *Gnot1* transcripts in the presence of cycloheximide also indicates the presence of a potent and labile intracellular inhibitor capable of modulating *Gnot1* expression. During gastrulation, *Gnot1* transcripts become localized specifically to tissues associated with "organizer" function (Hensen's node, head process, notochord). The expression data and the response to mesoderm inducing factors and axial "caudalizing" signals suggest that *Gnot1* may be involved in specification of the embryonic body axis and could play a part in regulating features of the trunk/tail organizer in the chick embryo.** © 1995 Academic Press, Inc.

INTRODUCTION

During gastrulation, a bilaminar amniote embryo (or blastoderm) consisting of primary ectoderm (epiblast) and primitive endoderm (hypoblast) is converted into a trilaminar structure (ectoderm, mesoderm, and definitive endoderm) with a defined rostrocaudal, or anteroposterior (AP), axis. The formation of the AP axis results from the action of certain "organizing" centers (reviewed by Stern *et al.*, 1992; Khaner, 1993; Kessler and Melton, 1994). The most extensively studied, Hensen's node, forms during gastrulation and is considered the equivalent of the amphibian Spemann's organizer (reviewed by Leikola, 1976; Streit *et al.*, 1994). Functional studies in the chick suggest that, as in amphibians, organizing centers are already present well before the onset of gastrulation (reviewed by Khaner, 1993). In early pregastrulation embryos with a single epiblast layer, axis-determining properties are associated with the posterior marginal zone (PMZ) of the embryo (Eyal-Giladi and Spratt, 1965). Later, these properties become associated with the secondary hypoblast (Waddington, 1933; Azar and Eyal-Giladi, 1981; Eyal-Giladi *et al.*, 1994), which arises posteriorly from the PMZ and extends anteriorly with time to form a complete layer beneath the epiblast just prior to gastrulation (Eyal-Giladi and Kochav, 1976).

It is still not clear what the endogenous mediators of organizer action are. However, several types of signaling molecules are able to experimentally affect primary axis formation in vertebrate embryos, suggesting that they may also act as physiological mediators (see reviews by Jessel and Melton, 1992; Stern *et al.*, 1992; Kessler and Melton, 1994). Among these are growth factors of the TGF-β and FGF families, which are able to induce primary mesoderm in *Xenopus*, and retinoic acid, which can alter axial pattern and affects expression of many homeobox genes (Kimelman and Kirschner, 1987; Slack, 1987; Durston *et al.*, 1989; Thomsen *et al.*, 1990; review by Krumlauf, 1994). Activin and FGF are present in the early pregastrulation chick em-

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bryo when "early" organizing centers are signaling (Mitrani *et al.*, 1990a,b), and their distribution and action under certain experimental conditions suggest that they may play a role in this process (Mitrani *et al.*, 1990b; Ziv *et al.*, 1992; Cooke *et al.*, 1994; Gordon-Thomson and Fabian, 1994). While the role of endogenous retinoids in primary embryonic induction is as yet uncertain, retinoic acid has been detected in gastrulating chick embryos (Chen *et al.*, 1992) and experimentally applied retinoic acid is known to modulate axis formation, having a profound ability to caudalize (or posteriorize) the embryonic axis in vertebrates (Durston *et al.*, 1989; Kessel and Gruss, 1991; Modak *et al.*, 1993).

One obvious outcome of the activity of such early signaling mediators will be to alter the program of gene expression in responding cells. Several types of transcription factors have been specifically associated with "late" organizer cells and their derivatives in vertebrates; these include members of the *Forkhead/HNF, Brachyury*, and homeobox gene (Not, LIM, *goosecoid*) families (Cho *et al.*, 1991; Dirksen and Jamrich, 1992; Ruiz i Altaba and Jessel, 1992; Taira *et al.*, 1992; Gont *et al.*, 1993; von Dassow *et al.*, 1993; reviewed by Herrmann and Kispert, 1994; Streit *et al.*, 1994). Although natural mutants and/or molecular genetic approaches have implicated some of these genes in the regulation of organizer specification and/or signaling functions (Cho *et al.*, 1991; Ang and Rossant, 1994; Taira *et al.*, 1994b; Weinstein *et al.*, 1994; Shawlot and Behringer, 1995; review by Herrmann and Kispert, 1994), the roles of others, such as members of the Not homeobox family, remain to be determined.

Among amniotes, the chick model has the advantage of easy access to pregastrulation stage embryos for analyses and manipulation and the capability to genetically alter gene expression (see for example Nieto *et al.*, 1994). However, relatively few organizer-associated regulatory genes have been isolated and analyzed in the chick (*Cnot, goosecoid*) and only *goosecoid* has been evaluated in pregastrulation embryos (Izpisua-Belmonte *et al.*, 1993; Stein and Kessel, 1995). We previously isolated a chick member of the Not family of homeobox genes (*Gnot1*, Ranson *et al.*, 1995), expressed selectively in the developing limb and in the developing tail notochord during midembryogenesis. The latter site of expression prompted us to evaluate the potential involvement of this gene in gastrulation. In this report, we have analyzed the expression of *Gnot1* prior to and during gastrulation and evaluated the regulation of *Gnot1* expression in cultured primary blastoderm cells by several types of signaling factor capable of affecting both primary axis formation and organizer-specific gene expression.

MATERIALS AND METHODS

Embryos

White Leghorn chick embryos were incubated at 38°C and staged as described by Eyal-Giladi and Kochav (EG&K: roman numerals) for pregastrulation stages and by Hamburger and Hamilton (HH: arabic numerals) for all later embryos (Hamburger and Hamilton, 1951; Eyal-Giladi and Kochav, 1976). Embryos were dissected in phosphate-buffered saline (PBS) and processed for experiments as described below.

Preparation and Analysis of RNA

Total RNA was extracted from embryonic chick tissues using ULTRASPEC RNA (Biotecx Labs, Inc.) as suggested by the manufacturer and Northern blots were prepared and hybridized with either random-primed DNA probes or antisense riboprobes using standard methods (Sambrook *et al.*, 1989). Hybridization was either carried out in 50% formamide as previously described (Mackem and Mahon, 1991), except that the temperature was increased to 77°C for riboprobes, or carried out in Rapid-Hyb buffer (Amersham) as recommended by the manufacturer for DNA probes. A chick β-actin probe control was used as described (Mackem and Mahon, 1991).

Isolation and Analysis of cDNA Clones

To analyze alternate *Gnot1* transcripts during gastrulation, a Lambda ZAP II (Stratagene) cDNA library from stage 5–9 chick embryos was screened with an end-labeled 60-base-long probe ($^{5'}$AGTTGTCTCACTGCACAGGGGCCG ACCCGCTGGGCCCTGCTGTGCTGTGGAGGTCTG-GAG$^{3'}$) corresponding to *Gnot1* sequence just 5′ of the homeobox, under conditions previously described (Mackem and Mahon, 1991). The partial sequence of a number of cDNA clones was determined by dideoxy sequencing (Sanger *et al.*, 1977) and dot-blots of clones were hybridized with random-primed ^{32}P-labeled DNA probes (shown in Fig. 1) using standard methods (Sambrook *et al.*, 1989). Exon 2 and intron 3 probes were generated using the polymerase chain reaction (PCR; Perkin–Elmer reagents and conditions), and clones were evaluated for the presence of exon 3 sequences by analytical PCR. The nucleotide sequence of genomic *Gnot1* clones (described in Ranson *et al.*, 1995), including the complete intronic regions, were determined and deposited with GenBank (Accession No. U23054).

Whole Mount in Situ Hybridization of Embryos

A Bluescript subclone containing the *PvuII–PstI* fragment from the 3′ untranslated region (3′ UTR) of *Gnot1* (Ranson *et al.*, 1995; see also Fig. 1C) was used to generate digoxigenin–UTP riboprobes as previously described. Embryos were prepared for whole mount *in situ* hybridization, hybridized, and washed and the hybrids visualized with alkaline phosphatase conjugated anti-digoxigenin, exactly as described by Conlon and Rossant (1992), except that the length of proteinase K treatment varied from 1 to 5 min depending on the size of the embryos. The color reaction was stopped, usually after 0.5–2 hr, and embryos were stored at 4°C in PBS containing 2.5 m*M* EDTA and 0.1%

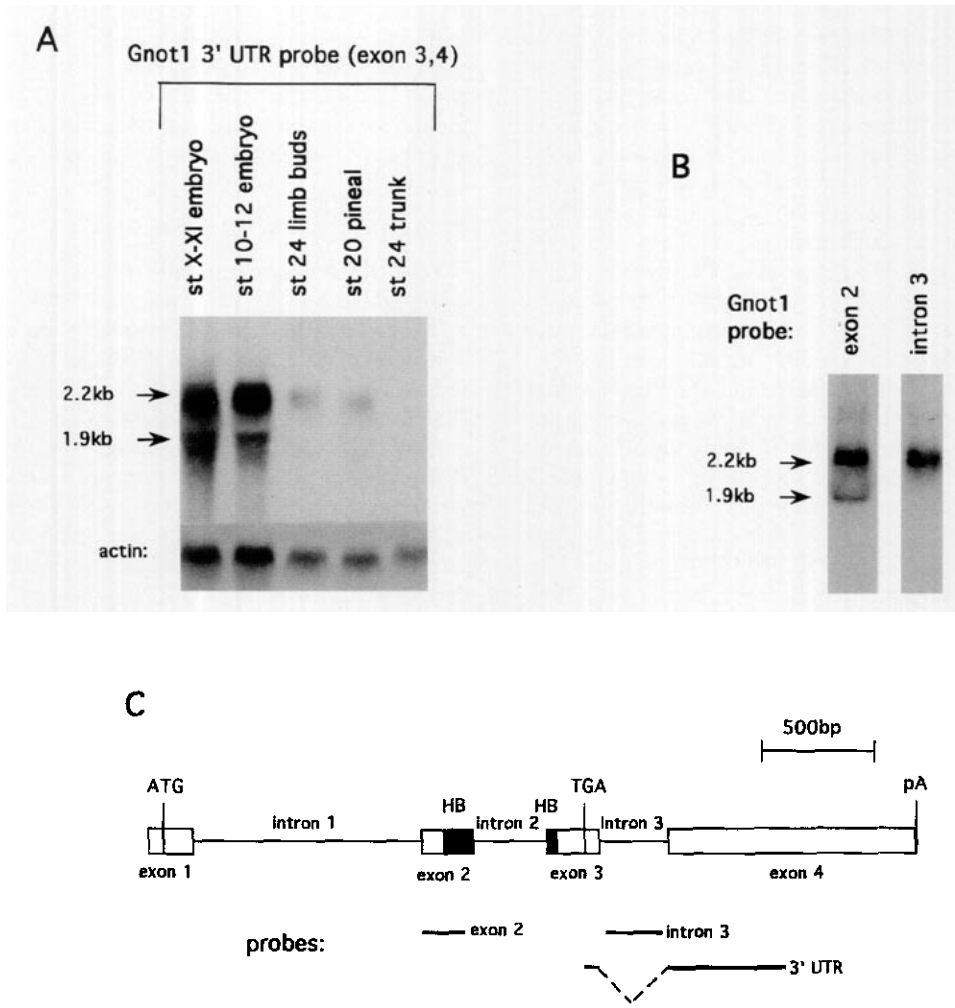

FIG. 1. Northern blot analysis of expression of alternate *Gnot1* transcripts. Ten micrograms of total RNA per lane was separated on 1% formaldehyde gels and hybridized to probes shown schematically in (C). Transcript size was assessed using a BRL RNA ladder. The positions of the 2.2- and 1.9-kb *Gnot1* transcripts are indicated. (A) Profile of developmental expression of *Gnot1*. Total RNA was isolated from whole embryos or from dissected tissues at the stages indicated (limbs and tail were removed from "trunk"). Blots were hybridized with the *Gnot1* 3' untranslated region probe (3' UTR), stripped, and rehybridized with a chick β-actin probe, as shown, to control for loading. (B) Comparison of alternate *Gnot1* transcripts for the presence of intron 3. The same blot, loaded with pregastrulation (stages X–XI) RNA, was hybridized with an exon 2 probe, stripped, and rehybridized with an intron 3 probe. (C) Diagram of *Gnot1* genomic structure showing arrangements of exons and introns and the different probes used.

Tween 20 and photographed without clearing. Stained embryos were processed for histological sections as described by Izpisua-Belmonte *et al.* (1993).

Blastoderm Cell Culture

A suspension of blastoderm cells was obtained as described (Mitrani and Eyal-Giladi, 1982). Cells were cultured in DMEM with 10% FCS at 38°C. For short incubation times (6 hr) cells were agitated in suspension. For longer times (12–24 hr) cells were plated onto culture dishes, achieving about 30–40% confluency. About 10 dissociated blastoderms were seeded per 35-mm culture dish or per 1-ml shaking culture (yielding about 15 μg of total RNA at 24 hr). Additions to the cultures were as follows: bFGF (Becton Dickinson) or FGF-4 (gift from Genetics Institute, Inc.) at 150 ng/ml up to 1000 ng/ml together with 100 ng/ml of heparin sulfate (Sigma); activin A (BioSource International, Inc.) at 10, 20, and 50 U/ml with units of activity determined by the manufacturer using the *Xenopus* animal cap assay; all-*trans* retinoic acid (Sigma) at 10^{-7} to 10^{-5} *M*; and cycloheximide (Sigma) at 5 μg/ml. Where cycloheximide was used, cells were pretreated with cycloheximide for 2 hr prior to the addition of growth factors or retinoic acid. Under these conditions, a 95% inhibition of protein synthesis was achieved (based on relative incorporation of [^{35}S]-

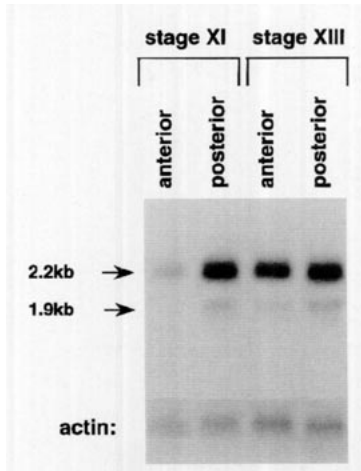

FIG. 3. Northern blot analysis of distribution of *Gnot1* transcripts within anterior and posterior halves of early and late pregastrulation embryos. Total RNA was isolated from separated anterior and posterior halves of stage XI (early) and of stage XIII (late) pregastrulation blastoderms, electrophoresed on 1% formaldehyde gels, blotted, and hybridized with the 3′ UTR probe (shown in Fig. 1C). The 2.2- and 1.9-kb *Gnot1* transcripts are indicated by arrows. Blots were rehybridized with a chick β-actin probe, shown below, to control for loading.

methionine and cysteine into total protein). In the absence of any added factors, the baseline expression of *Gnot1* RNA in cultured cells gradually declined between 12 and 24 hr compared to time zero.

RESULTS

Gnot1 Is Expressed as Two Transcripts in the Early Embryo

Gnot1 expression was easily detected by Northern blot analysis of total RNA from early pregastrulation (stages X–XI) and postgastrulation (stages 10–12, several somites) embryos (Fig. 1A), as compared to midembryogenesis times, when expression was restricted mainly to very localized regions in the limb buds and to the pineal (Ranson *et al.*, 1995). At these early stages, two *Gnot1* transcripts were readily apparent (Fig. 1A), a major 2.2-kb and a minor 1.9-kb transcript. Both transcripts were sense strand and were entirely poly(A)$^+$ (data not shown). The longer transcript, however, retained a 320-base intron located in the 3′ untranslated region of the gene (intron 3, Figs. 1B and 1C). To estimate the relative abundance of the short and long transcripts in early embryos, a number of *Gnot1* clones isolated from a stage 5–9 cDNA library that were both exon 3 and exon 4 positive were screened for the presence of intron 3, and 80% of these (44/55) retained intron 3 sequences. The detection of two differently processed transcripts was of interest in relation to additional experiments which suggested negative posttranscriptional regulation of *Gnot1* expression (see below).

Gnot1 Expression in the Pregastrulation Blastoderm and during Early Gastrulation

The spatial distribution of *Gnot1* transcripts during early embryogenesis was analyzed by whole mount *in situ* hybridization using an antisense digoxigenin–UTP-labeled riboprobe generated from the 3′ untranslated region (3′ UTR in Fig. 1C). The control sense riboprobe gave no detectable hybridization signal at any stages analyzed (data not shown).

The chick blastoderm in a freshly laid egg (stage X) consists of a single cell layer epiblast in the central embryonic part (area pellucida), encircled by a ring of denser extraembryonic tissue (area opaca). The lower hypoblast layer then arises from a dual origin, both as a sheet extending from precursor cells located at the posterior border (posterior marginal zone) of the embryonic blastoderm and from small groups of cells ingressing directly downward from the epiblast to form the primary hypoblast islands (reviewed by Khaner, 1993). Once the secondary hypoblast is complete (stage XIII), the chick embryo is comparable to the amphibian blastula or mammalian (nonrodent) blastocyst, with a blastocoel cavity and with a lower hypoblast layer analogous to the frog vegetal pole or mammalian primitive endoderm.

A weak, uniform *Gnot1* hybridization signal was seen throughout the area pellucida of stage X embryos (data not shown), which increased in intensity in slightly older blastoderms (stages XI–XII), particularly in that part of the area pellucida where the hypoblast sheet had already formed (as diagrammed in Fig. 2A and shown in Fig. 2B parts A–D). Transverse sections showed that the most intense staining was localized within the epiblast layer at these stages (Fig. 2B parts I and J). Occasional scattered cells of the posteriorly arising hypoblast layer and detached "middle cells" were also stained (Fig. 2B part J), as were rare cells scattered in the area opaca (not shown). No staining was detected in cells of the anteriorly located primary hypoblast islands (Fig. 2B part I). Thus, from stages X–XII, *Gnot1* transcripts increased in a stepwise fashion in the epiblast, beginning from posterior to anterior, that corresponded with the extent of the underlying, anteriorly migrating hypoblast layer at each stage (Figs. 2A and 2B). A correlation between stimulation of *Gnot1* expression and ingrowth of the hypoblast was also confirmed by Northern blot analysis (Fig. 3). At an early stage (XI), when only the posterior half of the embryo was in contact with a hypoblast layer, *Gnot1* expression was much higher in the posterior than in the anterior half of the embryo. After the formation of the hypoblast layer was complete (stages XIII–XIV), the *Gnot1* hybridization signal was more uniformly distributed throughout the area pellucida (Fig. 2B parts E–H and Fig. 3). This uniform hybridization signal declined with the first appearance of localized *Gnot1* expression at the onset of gastrulation.

With the formation and elongation of the primitive streak

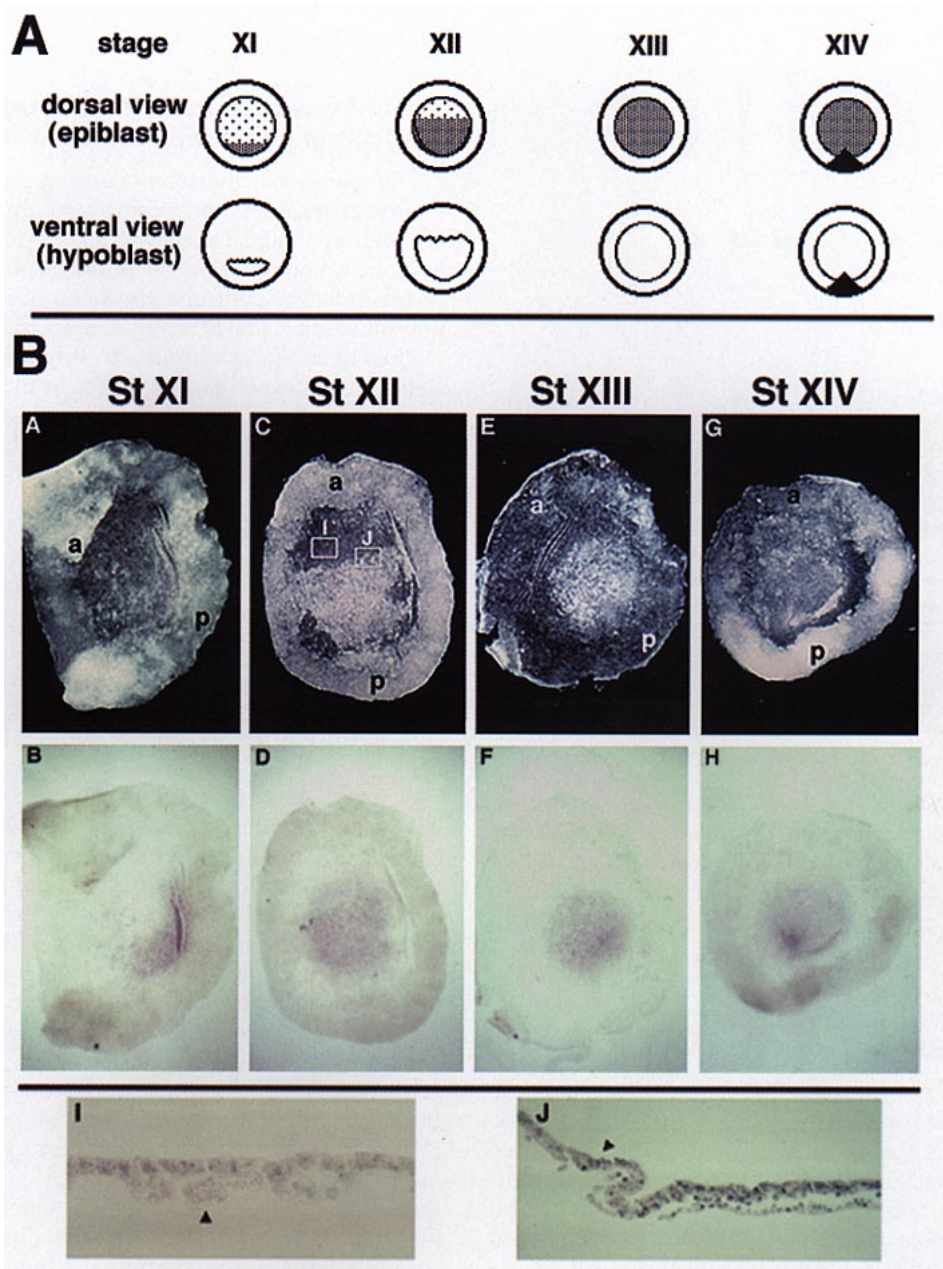

FIG. 2. Spatial distribution of *Gnot1* mRNA in pregastrulation chick embryos. (A) Schematic summary showing formation of the hypoblast sheet (ventral layer) and corresponding changes in expression of *Gnot1* (stippling) in the dorsal epiblast layer. The density of the stippling indicates relative expression level (low or high) of *Gnot1*. Blastoderm diagrams are oriented with anterior border at the top. (B) Dark- and bright-field images of the same embryo are shown in pairs to illustrate morphology and staining after *in situ* hybridization with *Gnot1* probe, respectively. Stages (EG&K) are indicated above. The orientation of embryos with respect to anterior (a) and posterior (p) borders is indicated on dark-field image. Transverse sections (I and J) were taken from the boxed regions of the embryo shown in C. Note that staining due to *Gnot1* expression, predominantly in the epiblast, is closely correlated with the extent of underlying hypoblast formation arising from the posterior marginal zone. Anteriorly located primary hypoblast islands (arrowhead in I) show no signal and the overlying epiblast shows very weak hybridization for *Gnot1*. As the posteriorly derived hypoblast sheet extends under the epiblast, *Gnot1* expression increases in the overlying epiblast (arrowhead in J indicates transition point in epiblast where underlying hypoblast sheet ends). Occasional detached middle cells and cells of the hypoblast sheet also show expression of *Gnot1*.

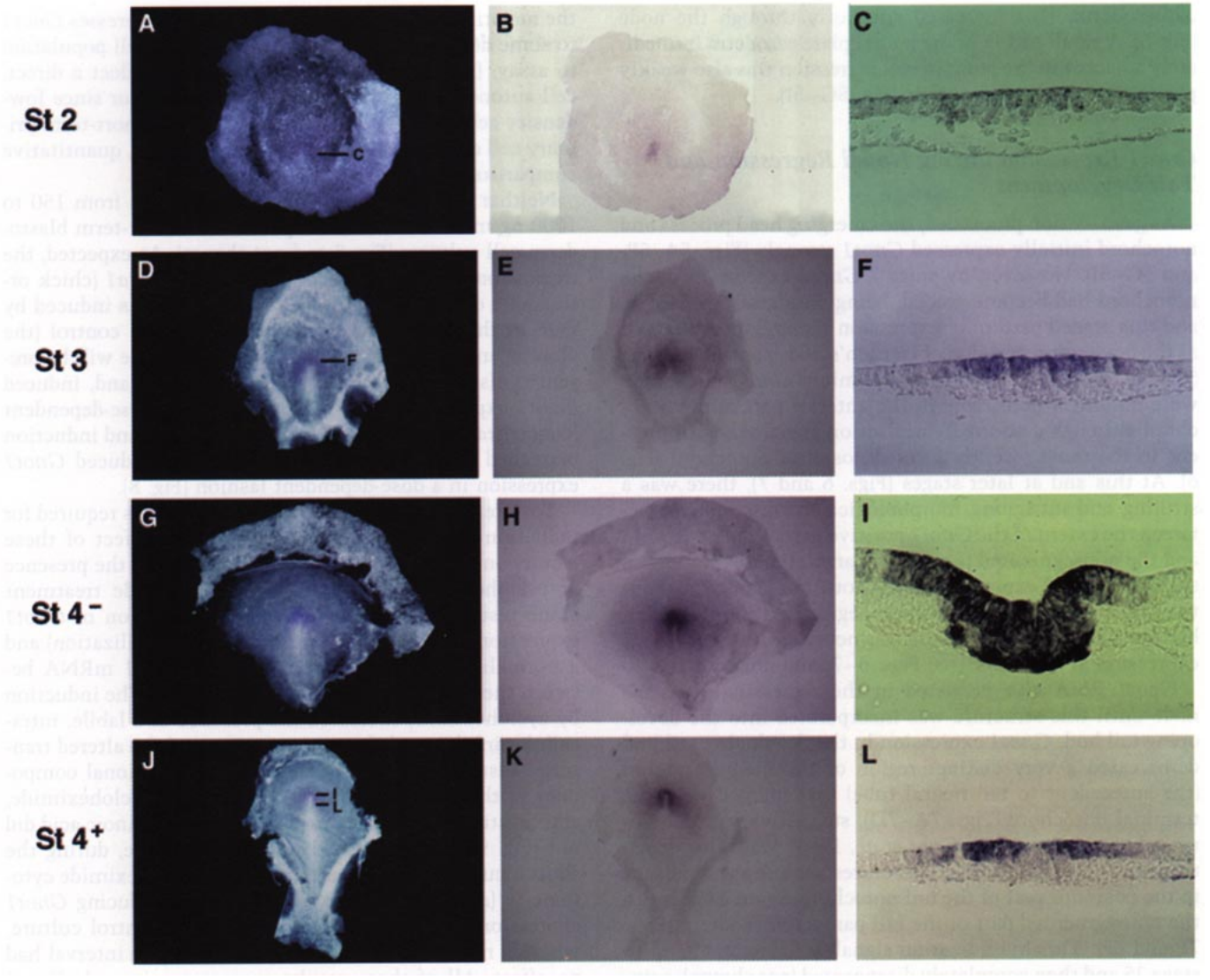

FIG. 4. Localization of *Gnot1* mRNA during early gastrulation. Dark- and bright-field images of the same embryo are shown in pairs, with the stage (HH) indicated to the left. Transverse sections (C, F, I, and L) were taken at the level indicated from the central region of embryos in A, D, and J. Strong staining due to *Gnot1* transcripts is found in the region of the forming "late" organizer (anterior edge of the primitive streak and then Hensen's node). Ectodermal staining extends along either side of the anterior-most primitive streak as well. A weak signal is also present in an arc extending posterolaterally around either side of the node.

(stages 2–4), *Gnot1* mRNA became predominantly localized to epiblast cells at the anterior end of the forming primitive streak and was lost completely from the hypoblast (Fig. 4). Expression in the anterior streak region of the epiblast was initially broad and diffuse (Figs. 4A–4F) and gradually became restricted to the forming Hensen's node in a horseshoe pattern at the anterior edge of the primitive streak by stage 4 (Figs. 4G–4L). Sections of Hensen's node revealed that all cells of the region just anterior to the primitive pit (where the three germ layers are contiguous) strongly expressed *Gnot1* (Fig. 4I). Just posterior to the pit, cells of the primitive streak did not express *Gnot1* and the strong staining along each side of the streak was exclusively ectodermal (Fig. 4L). Between stages 2 and 4, a low level of staining due to *Gnot1* hybridization was also apparent throughout the entire anterior half of the epiblast (Fig. 4) and gradually disappeared by about stage 5 except for a residual convex arc-like area extending postero-laterally from either side of Hensen's node (Figs. 4J, 4K, and 5A–5D), which persisted until about stage 10–11. As gastrulation proceeded, the strong *Gnot1* hybridization seen in Hensen's node also persisted in the direct nodal descendant, the chor-

damesoderm, that ingressed anteriorly through the node (Fig. 5). A small region of the neural plate ectoderm immediately adjacent to the point of cell ingression was also weakly positive for *Gnot1* transcripts (Fig. 5G–5I).

Gnot1 Expression during Nodal Regression and Tail Development

As gastrulation proceeded, the emerging head process and notochord initially expressed *Gnot1* strongly (Figs. 5A, 5B, and 5G–5I). However, by stage 7 *Gnot1* expression in the notochord had become graded, being strongest posteriorly, and this graded pattern of expression became more marked as the posterior regression of Hensen's node continued (Figs. 5C–5F). After stage 10 (about 10 somites) *Gnot1* transcripts were no longer detectable in the anterior part of the notochord although a strong hybridization signal was still present in the most recently formed posterior notochord (Fig. 6). At this and at later stages (Figs. 6 and 7), there was a striking and intriguing morphological correspondence between the extent of the *Gnot1* positive part of the notochord and the nonsegmented part of the paraxial plate mesoderm, such that *Gnot1* expression in the notochord ended at about the position of the most recently segmented somite. Notably, the neural tube floorplate was never positive for *Gnot1* expression at any stage (see Figs. 5–7, and not shown).

Gnot1 RNA also persisted in the regressing Hensen's node until this structure was incorporated into the developing tail bud. *Gnot1* expression in the developing tail bud demarcated a very distinct region of the medullary cord (the antecedent to tail neural tube) that merged with the terminal notochord (Figs. 7A–7D), suggestive of a chordoneural hinge equivalent (Gont *et al.*, 1993). With the formation of the tail and its axial structures, staining was present in the posterior part of the tail notochord, again adjacent to the nonsegmented part of the tail paraxial mesoderm (Figs. 7E and 7F). This hybridization signal was detectable up to stage 25 and then completely disappeared (not shown), coincident with the onset of tail degeneration in the chick (Schoenwolf, 1981).

Beginning at about stage 16, a strong *Gnot1* hybridization signal was present in the epiphysis/pineal (Figs. 7A and 7C). Prior to formation of the epiphysis anlage, a very weak hybridization signal was also detectable in the anterior neural folds near the anterior neuropore (data not shown).

Regulation of Gnot1 Expression in Blastoderm Cells

The finding of *Gnot1* expression in regions of the chick embryo known to have organizer properties and the pattern of blastodermal expression suggesting activation by signals from the hypoblast prompted us to investigate the effect of known signaling factors on *Gnot1* expression. To analyze the effect of such factors on *Gnot1* expression, a short-term primary culture, derived from trypsinized pregastrulation blastoderm (stages X–XII), was used for several reasons: (1) the majority of cells at this stage normally expresses *Gnot1* to some degree and so provide a responsive cell population to assay; (2) the result would be likely to reflect a direct, cell autonomous response to a particular factor since low-density cell culture was used; (3) the use of short-term primary cell cultures facilitated easily controlled, quantitative comparisons.

Neither bFGF nor FGF-4 at concentrations from 150 to 1000 ng/ml altered *Gnot1* expression in short-term blastoderm cell cultures (Fig. 8 and not shown). As expected, the expression of the organizer-specific gene *Gbra1* (chick orthologue of the mouse *Brachyury/T* gene) was induced by FGF in the same cells, serving as a positive control (the cloning and characterization of the *Gbra1* gene will be presented elsewhere). Activin A, on the other hand, induced *Gnot1* expression (Fig. 8). This effect was dose-dependent (concentrations below 10 U/ml had no effect and induction plateaued at 50 U/ml). Retinoic acid also induced *Gnot1* expression in a dose-dependent fashion (Fig. 8).

To determine whether protein synthesis was required for induction by activin or retinoic acid, the effect of these factors on *Gnot1* expression was examined in the presence of cycloheximide (Fig. 9). The cycloheximide treatment alone resulted in both a tremendous induction of *Gnot1* expression (due to either derepression or stabilization) and a normalization of the distribution of *Gnot1* mRNA between the smaller and the larger transcripts. The induction by cycloheximide indicated the presence of a labile, intracellular inhibitor of *Gnot1* expression, and the altered transcript distribution suggested a posttranscriptional component in this regulation. In the presence of cycloheximide, activin stimulated *Gnot1* expression while retinoic acid did not (Fig. 9). FGF again had no effect. Of note, during the short incubation times necessitated by cycloheximide cytotoxicity (about 6 hr), activin was already inducing *Gnot1* expression in the cycloheximide-negative control culture, whereas retinoic acid alone at this short time interval had no effect. All of these results are consistent with *Gnot1* being an immediate-early response gene to activin (and potentially to mesoderm induction) and a delayed or indirect response gene to retinoid signals.

Since embryonic axis induction *in vivo* results from multiple, potentially combinatorial signals, these signaling factors were also applied to blastoderm cultures in various combinations. A strong synergistic effect between retinoic acid and activin was observed in the induction of *Gnot1* expression (Fig. 10).

DISCUSSION

Recent work in a number of systems suggests that many of the same regulatory and signaling cascades may be employed in the formation and development of the primary embryonic field (body axis) and secondary embryonic fields (e.g., organogenesis, limb development) (see for example Jessel and Melton, 1992; Gaunt *et al.*, 1993; Kessler and Mel-

ton, 1994; Maden, 1994). We previously analyzed the expression pattern and possible role of the chick homeobox gene *Gnot1* in establishment of one of the secondary fields (limb development, Ranson *et al.*, 1995). The expression of this gene in caudal notochord during midembryogenesis prompted us to analyze its potential involvement in gastrulation.

Pre- and Perigastrulation Expression of Gnot1 and Relation to the Organizer

Before gastrulation, *Gnot1* expression in the epiblast is upregulated as the posteriorly arising hypoblast comes to reside beneath the epiblast layer, suggesting an inductive interaction mediated by the hypoblast. At the onset of gastrulation, this broad domain of *Gnot1* expression is extinguished and replaced by a more localized domain in the forming Hensen's node. The expression of *Gnot1* before gastrulation is very reminiscent of the expression of its *Xenopus* relative, *Xnot*, and rather different from that of a proposed "head" organizer gene, *goosecoid*. *Xnot*, which has been proposed as a component of a trunk/tail organizer, is uniformly expressed in the *Xenopus* blastula at the onset of zygotic transcription and this expression is progressively lost from areas outside of the organizer (dorsal blastopore lip) as gastrulation proceeds (von Dassow *et al.*, 1993). In contrast, *goosecoid* transcripts are highly localized even before gastrulation, in both *Xenopus* and the chick (Cho *et al.*, 1991; Izpisua-Belmonte *et al.*, 1993). The similar time of onset and the progression from diffuse to localized expression shared between *Gnot1* and *Xnot* suggest conserved roles during gastrulation. Likewise, the extinction of expression that occurs in cells outside the organizer region also indicates an important component of negative regulation operating in the early expression of both *Xnot* and *Gnot1* (discussed further below).

While Hensen's node is considered to be an equivalent of the amphibian organizer (reviewed by Leikola, 1976; Streit *et al.*, 1994), experimental organizer properties also extend to part of the anterior definitive primitive streak and the immediately adjacent lateral ectoderm (Waddington, 1932; Mulherkar, 1958; Storey *et al.*, 1995). *Gnot1* is not expressed within the primitive streak proper and so does not strictly correlate with all regions having organizing potential. *Goosecoid* shows a somewhat different pattern in the node, less evident in the lateral ectoderm, and later is expressed only more anteriorly, in prechordal plate (Izpisua-Belmonte *et al.*, 1993). Such heterogeneity of gene expression within the organizer is of potential interest in relation to recent evidence for regional functional differences within the chick organizer (see Storey *et al.*, 1995).

During primitive streak regression, a weak arc of ectodermal *Gnot1* expression is also present extending posterolaterally from the node (stages 5–10). Stein and Kessel (1995) examined the perigastrulation expression of *Gnot1* (or *Cnot*) and proposed that these cells will contribute to the spinal cord neuroectoderm, but fate maps indicate that cells in this ectodermal zone also contribute to the tailbud (Spratt, 1952; Stein and Kessel, 1995). Since *Gnot1* is expressed in the tailbud, this expression domain could also represent a population of cells that are still being recruited to Hensen's node, or its equivalent, in the tailbud.

Like many of the organizer-associated genes, *Gnot1* is also expressed in the notochord, a descendant of Hensen's node that is essential for patterning of somites and neural tube (see discussions by Ang and Rossant, 1994; Weinstein *et al.*, 1994; and references therein). Unlike some of these genes (e.g., *HNF, Xnot*), *Gnot1* is not expressed in the neural tube floorplate which, upon interaction with the notochord, also acquires signaling functions for dorsoventral patterning of the neural tube. This could suggest that *Gnot1* is an unlikely regulator of such signaling activities per se or that the regulatory pathways differ despite functional similarity between notochord and floorplate signals. *Gnot1* expression in the notochord is initially graded and later becomes localized exclusively in the posterior notochord adjacent to nonsegmented paraxial mesoderm. Some organizer-associated genes are expressed uniformly along the length of the notochord (e.g., *Brachyury, HNF*; Ang and Rossant, 1994; Herrmann and Kispert, 1994; Weinstein *et al.*, 1994). However, like *Gnot1, Xnot* and *Xlim1* also show a more posterior distribution of expression (Gont *et al.*, 1993; von Dassow *et al.*, 1993; Taira *et al.*, 1994a). Such nonuniform gene expression points to potential regional differences in notochord function. In the case of *Gnot1*, the correlation of expression level in the chordamesoderm with the level of transition from segmented to nonsegmented paraxial mesoderm suggests a role in regulating notochordal signals involved in the process of paraxial segmentation.

During midembryogenesis, *Gnot1* is also expressed in the developing tailbud. There has been an ongoing debate as to whether the development of tail structures in amniotes occurs via a continuation of gastrulation (involving a node-like organizer) or whether these structures arise directly from a multipotent blastema (Griffith *et al.*, 1992). In amphibians, where the former mechanism of tail development is favored, *Xnot* is expressed specifically in the chordoneural hinge region of the tailbud (Gont *et al.*, 1993; Tucker and Slack, 1995). *Gnot1* hybridization in the chick tailbud appears to highlight an equivalent structure; this gene may be of utility in analyzing tail development in amniotes.

Role of Mesoderm-Inducing Growth Factors, Retinoic Acid, and Inhibitor(s) in Gnot1 Regulation

Formation of the body axis involves two events: (1) the primary formation of mesoderm; and (2) the organization of newly formed mesoderm into axial structures with inductive properties. There is still considerable debate as to the nature, order of action, and relative importance of physiologic mediators of these events, even in amphibians (see for example reviews by Jessel and Melton, 1992; Kessler and Melton, 1994). Exogenous activin A and FGF members can induce mesoderm formation in chicks, as well as in *Xeno-*

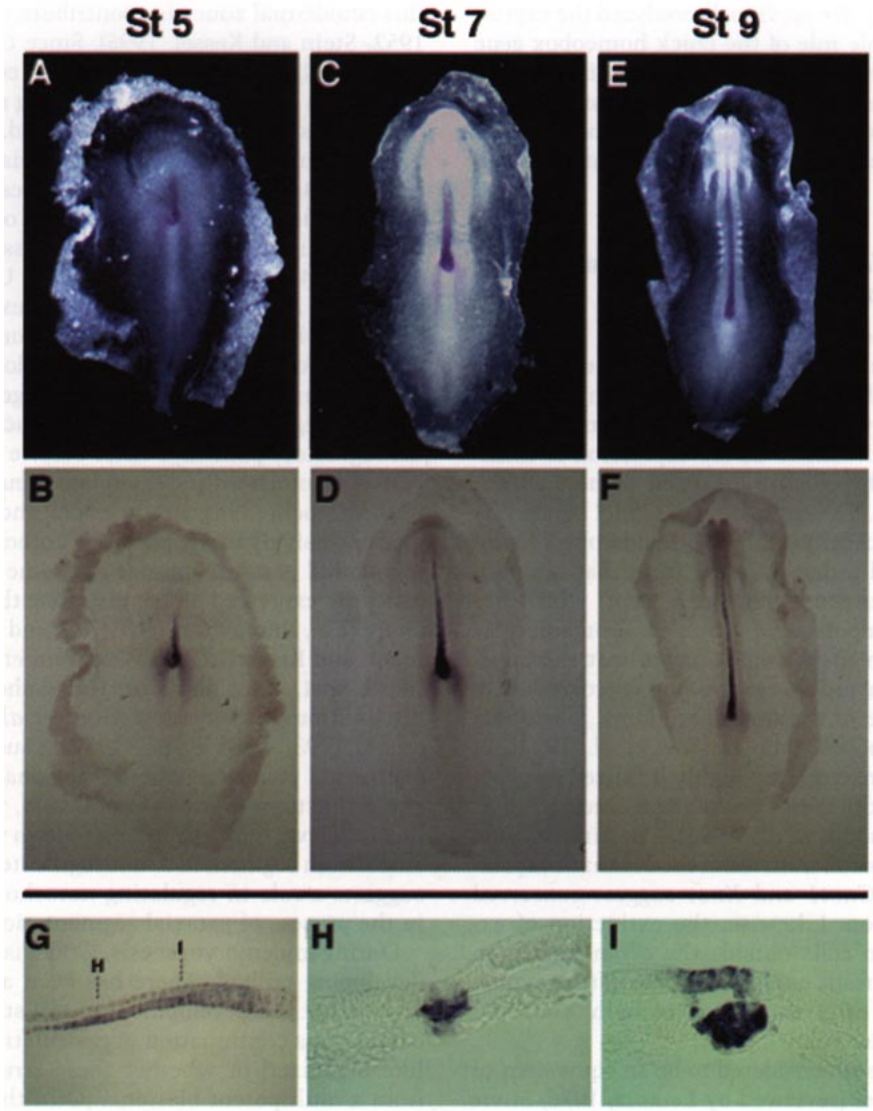

FIG. 5. Distribution of *Gnot1* mRNA during the regression phase of gastrulation. Dark- and bright-field images of the same embryo are shown in pairs, with the stage (HH) indicated above. The lower three panels (G–I) show a sagittal section (G) and transverse sections (H–I) at levels indicated in (G) from stage 5 embryos with staining similar to that of the embryo in A and B. The distribution of *Gnot1* transcripts is graded within the forming notochord, being highest posteriorly. Weak staining of the ectoderm in an arc posterolateral to the node is also visible. Sections demonstrate focal hybridization in the neuroectoderm immediately anterior to the node (G and I), as well as the graded expression of *Gnot1* in the notochord (H and I).

pus (Kimelman and Kirschner, 1987; Slack, 1987; Mitrani *et al.*, 1990b; Thomsen *et al.*, 1990; Ziv *et al.*, 1992; Gordon-Thomson and Fabian, 1994). Activin, which induces dorsal mesoderm (notochord), has the ability to induce secondary axis formation. Another intensively studied signaling factor, retinoic acid, has neither of these inductive properties, but can alter the polarity of an induced axis, promoting caudalization (Durston *et al.*, 1989; Kessel and Gruss, 1991; Modak *et al.*, 1993; reviewed by Jessel and Melton, 1992). The features of *Gnot1* expression suggest that it could play a role in regulating formation and/or function of tissues with organizing properties, and so it was of interest to evaluate the effects of these several types of extensively characterized factors on *Gnot1* expression.

Like many of the organizer-associated transcription factors that have been evaluated to date (Cho *et al.*, 1991; Dirksen and Jamrich, 1992; Taira *et al.*, 1992), *Gnot1* expression is induced by activin but not FGF and the induction by activin does not require *de novo* protein synthesis. Thus, *Gnot1* appears to be an immediate-early response gene to mesoderm induction. This is different than the regulation of *Xnot*. *Xnot* is unusual both in being responsive to

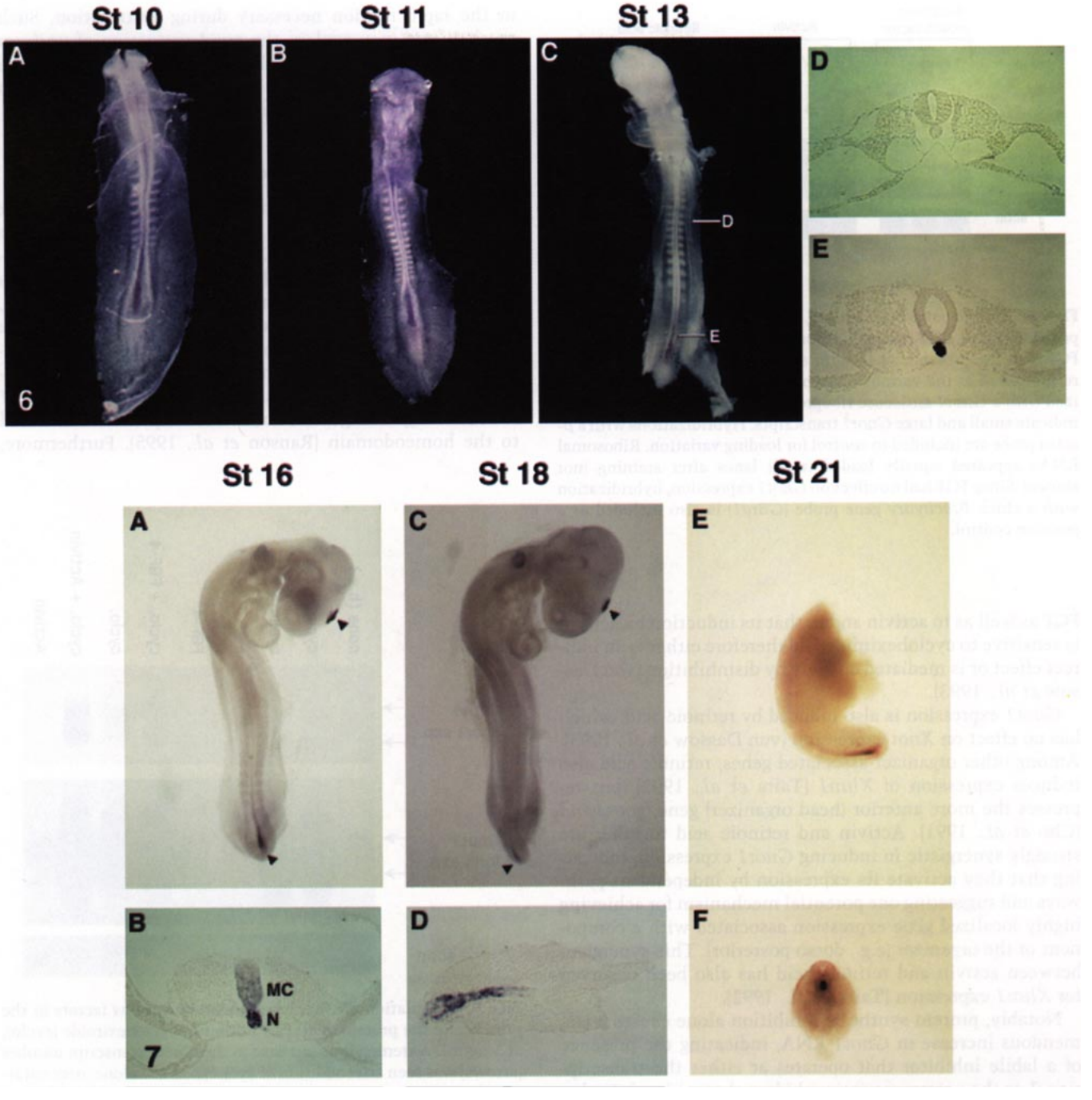

FIG. 6. Distribution of *Gnot1* transcripts during late gastrulation (midembryogenesis). Stages of embryos are indicated above. *Gnot1* is expressed in the most posterior levels of the notochord while expression in the anterior notochord is lost. Note that, at each stage, the *Gnot1* expressing region of the notochord ends at the level of most recently segmented (most posterior) somite. Transverse sections (D and E) at the levels indicated on the embryo in C show loss of staining in anterior notochord and absence of staining in neural tube floorplate.

FIG. 7. Distribution of the *Gnot1* transcripts in the developing tail. Stages of embryos are indicated above. Note *Gnot1* expression in the pineal gland and developing tail (arrows). (B) A transverse section through the tailbud of the panel A embryo; (D) a frontal section through the tail of the panel C embryo; and (E and F) sagittal (E) and transverse (F) sections of the dissected caudal trunk and tail of a stage 21 embryo. In the early tailbud (A and B), *Gnot1* is expressed in the region of the medullary cord (MC) as well as the terminal notochord (N), but not the adjacent tail paraxial mesoderm and mesenchymal cells. In older embryos (C–F), transcripts are found only in the notochord adjacent to the nonsegmented paraxial mesoderm at the tip of the tail.

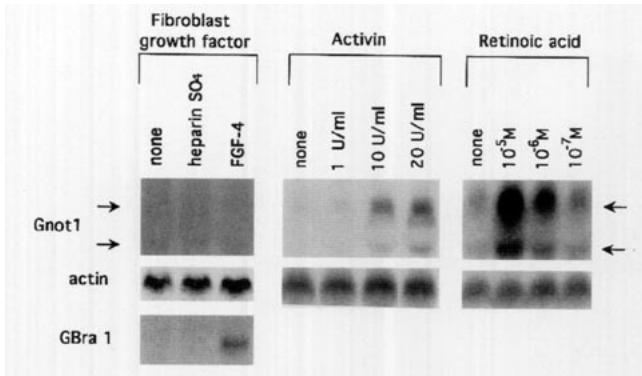

FIG. 8. Influence of FGF, activin, and retinoic acid on *Gnot1* expression. Total RNA extracted from cells treated with 200 μg/ml FGF-4 in the presence of heparin sulfate, with activin A, or with retinoic acid at the various concentrations indicated were hybridized with a *Gnot1* antisense riboprobe (3′ UTR in Fig. 1C). Arrows indicate small and large *Gnot1* transcripts. Hybridizations with a β-actin probe are included to control for loading variation. Ribosomal RNAs appeared equally loaded in all lanes after staining (not shown). Since FGF had no effect on *Gnot1* expression, hybridization with a chick *Brachyury* gene probe (*GBra1*) is also included as a positive control.

FGF as well as to activin and in that its induction by activin is sensitive to cycloheximide and therefore either is an indirect effect or is mediated entirely by disinhibition (von Dassow *et al.*, 1993).

Gnot1 expression is also induced by retinoic acid, which has no effect on *Xnot* expression (von Dassow *et al.*, 1993). Among other organizer-associated genes, retinoic acid also induces expression of *Xlim1* (Taira *et al.*, 1992), but represses the more anterior (head organizer) gene, *goosecoid* (Cho *et al.*, 1991). Activin and retinoic acid together are strongly synergistic in inducing *Gnot1* expression, indicating that they activate its expression by independent pathways and suggesting one potential mechanism for achieving highly localized gene expression associated with a component of the organizer (e.g., dorso-posterior). This synergism between activin and retinoic acid has also been observed for *Xlim1* expression (Taira *et al.*, 1992).

Notably, protein synthesis inhibition alone causes a tremendous increase in *Gnot1* RNA, indicating the presence of a labile inhibitor that operates at either the transcriptional or the posttranscriptional (degradation) level. In this respect, *Gnot1* regulation is like that of *Xnot*, which is also derepressed or stabilized by cycloheximide (von Dassow *et al.*, 1993). The presence of two *Gnot1* transcripts and the greatly increased relative abundance of the smaller, fully spliced form in the presence of cycloheximide suggest the importance of posttranscriptional mechanisms in mediating the negative regulation of *Gnot1*.

A combination of strongly synergistic inducing signals and potent mechanisms for transcript elimination could provide a means for modulating temporospatial expression in the rapid fashion necessary during gastrulation. Such mechanisms may explain the rapid extinction of uniform *Gnot1* expression in the epiblast at the onset of gastrulation and the subsequent highly restricted expression domain that is achieved in the organizer.

The Not Family of Homeobox Genes

There are at least two, structurally very different, chick homeobox genes containing a homeodomain that is most closely related to that of the *Xenopus* gene, *Xnot*, and genomic Southern blots suggest that the chick genome contains several additional related genes (Ranson *et al.*, 1995). Thus, this subfamily of homeobox genes may include multiple members displaying an organizer-associated expression pattern. *Gnot1* may not represent the "orthologue" of the *Xenopus* gene for several reasons. Structurally, *Xnot* and *Gnot1* are extremely divergent in amino acid sequence N-terminal to the homeodomain (Ranson *et al.*, 1995). Furthermore,

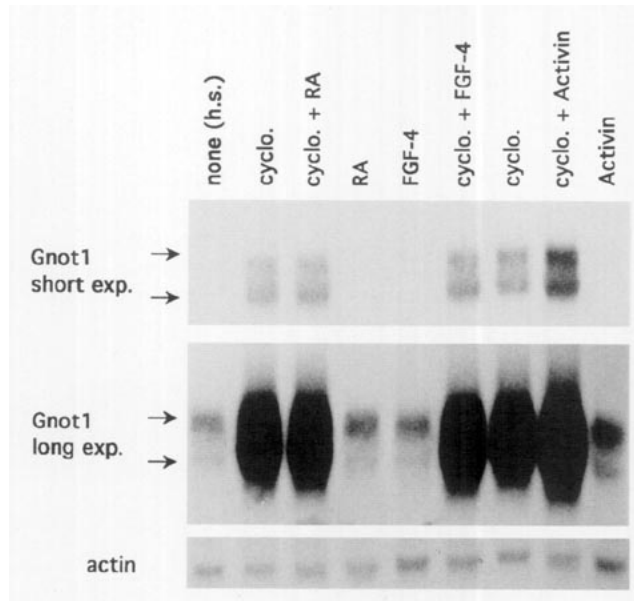

FIG. 9. Regulation of *Gnot1* expression by various factors in the presence of the protein synthesis inhibitor, cycloheximide (cyclo), at 5 μg/ml. A tremendous increase in the *Gnot1* transcript number (arrows) was seen after addition of cycloheximide alone, necessitating short exposures (top) to evaluate relative expression in cases where cycloheximide was added, and long exposures (middle) for samples in which cycloheximide was not included. Note also that the ratio of large to small *Gnot1* transcripts becomes 1:1 in the presence of cycloheximide as compared with about 5:1 elsewhere. Retinoic acid (RA, at 10^{-5} *M*) did not stimulate *Gnot1* expression above the level with cycloheximide alone, whereas activin A (at 50 U/ml) together with cycloheximide showed stimulation above the level of cycloheximide alone. Once again, FGF (200 μg/ml) showed no effect. A β-actin probe was again hybridized as a loading control, and ribosomal RNAs (not shown) also appeared equally loaded in all lanes after staining.

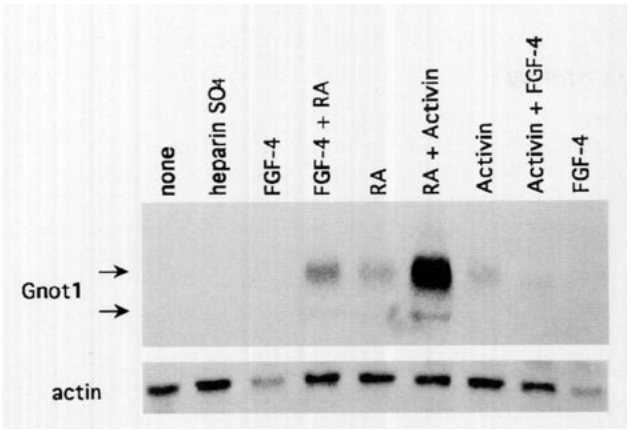

FIG. 10. Combinatorial effects of different factors on *Gnot1* expression. Factors at the same concentrations as those used in Fig. 9 (see legend) were applied alone or in combinations as indicated. A strong synergistic effect on induction of *Gnot1* expression (arrows) was seen when retinoic acid (RA) and activin were added together. Heparin sulfate (hs), which by itself never affected *Gnot1* mRNA levels, was included in the FGF-4 lane and in the "no additions" lane. A β-actin probe was again hybridized as a loading control, and ribosomal RNAs (not shown) also appeared equally loaded in all lanes after staining.

although there are remarkable similarities in expression and in certain aspects of regulation, *Gnot1* notably lacks the expression of *Xnot* in the neural tube floorplate and differs in many features of its regulation (von Dassow *et al.*, 1993). Recently, Stein and Kessel (1995) have reported on the peri- and postgastrulation expression of *Gnot1* (which they have named *Cnot*) beginning with stages 3–4 (early definitive streak). Our results on *Gnot1* expression at these later stages are overall in agreement with theirs.

The early onset of *Gnot1* expression prior to gastrulation with apparent activation by the hypoblast, its subsequent close association with regions of organizer activity (Hensen's node, head process, notochord), and its responsiveness to signals capable of inducing or modulating axis formation are all compatible with a role in regulating aspects of organizer formation and/or function during gastrulation. The development of gain-of-function and loss-of-function models will aid in determining the involvement of this gene and its relatives in gastrulation, but may be made more complex by the existence of several such related genes that could overlap functionally.

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